

Proposal for a Short-Term Toxicity Test with *Artemia* Nauplii

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Although standardization of toxicity tests on aquatic organisms is an urgent necessity, very little has yet been achieved for the marine environment. As a first step in this direction, a simple, inexpensive and reliable short-term routine test with *Artemia* larvae is proposed. This test is the result of an extensive study in our laboratory, taking into account the advantages and disadvantages of *Artemia* bioassays published by various authors. The major advantage of the brine shrimp as a test species is its continuous availability under the form of dry cysts which can be hatched very easily; this eliminates all biological, technological, and financial problems of stock recruitment and/or culturing. The acute test presented is based on the determination of the LC₅₀-24 hr of instar II-III nauplii of a specific *Artemia* strain. Presently this test is the subject of an intercalibration exercise in North America; an analogous exercise is now in progress in the European Economic Community countries.

INTRODUCTION

The necessity for the standardization of toxicity tests on aquatic organisms and the need for simple, inexpensive, routine tests have already been emphasized on many occasions, not the least at governmental levels.

Standardized tests with algae, crustaceans, and fishes are now close to being adopted at the international level for the freshwater environment. Despite the same urgency, very little has, however, been achieved for the marine environment.

For this reason we have tried to work out a simple, inexpensive, and reliable short-term routine test with *Artemia*, as a first reference yardstick, with unlimited applicability throughout the world.

For various reasons the brine shrimp is a uniquely suitable test species for laboratory experiments. Its cysts, indeed, can be stored for years under dry conditions without losing their viability and, upon immersion in seawater, the free-swimming nauplii will hatch out within approximately 24 hr.

The advantages of *Artemia* as the best "first choice" for toxicity studies in the marine environment can be summarized as follows:

- the cysts are commercially and readily available so that the tests can be carried out worldwide with the same original material and without any problem of provisioning; moreover, the quantity of cysts required per test is very small so that the price of the biological material is negligible;
- the necessity of year-round maintenance of stock cultures, with all the bio-

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logical and technical difficulties and the considerable economic repercussions, is completely eliminated;

—large numbers of test organisms of exactly the same age and physiological condition can be easily obtained to start the tests.

It has been objected by some that *Artemia* is not a “marine” organism. Let us emphasize, however, that *Artemia* has an extreme euryhaline character; its tolerance to salinity indeed ranges from brackish water to saturated brines! Brine shrimp are not encountered in the sea, because they are heavily predated (and completely eliminated) by invertebrates and fish. Quoting from one of our recent papers (Persoone and Sorgeloos, 1980): “. . . as a general rule we may say that the lowest salinity level in which *Artemia* is found, varies from place to place and is determined by the upper salinity tolerance level of the local predator. . . . As a result brine shrimp are very seldom found in waters with a salinity lower than 45‰, although physiologically they would feel perfectly at ease even in brackish waters. . . .” Last but not least brine shrimp are cultured worldwide—for fundamental research purposes as well as for aquaculture applications—in seawater of “normal” salinity (35‰), producing offspring which can be cultured further without any problem for many generations.

Although during the last two decades, a number of papers have been published on the toxic effects of some chemicals on brine shrimp, and methods have been proposed for short-term testing of chemicals, the existing literature revealed that there is no uniformity in the methodologies used. Because, in many cases, the experimental conditions were not described properly and little attention was paid to some important parameters influencing the results and the repeatability of the tests, intercomparison of the results is hardly possible.

Starting from the pertinent literature information on the use of *Artemia* as a test species for toxicity studies and literature data on the factors influencing the hatching and molting of brine shrimp (see review in Vanhaecke *et al.* (1980)) the following list of criteria was drafted as a basis for the development of a standard *Artemia* toxicity test with a reliable reproducibility:

- the nauplii have to be hatched out under strictly controlled conditions of temperature, salinity, aeration, light, and pH;
- the larvae must be of exactly the same age at the start of every test;
- during the test the larvae may not molt into an instar stage with a different sensitivity;
- the tests have to be carried out with cysts from the same geographical origin;
- the experimental conditions of the tests must be standardized and followed in every detail;
- a control test with a reference toxicant chemical must be carried out each time in parallel to check both the sensitivity of the larvae and the conformity with the standard technological procedure.

Starting from these criteria we studied the parameters of importance for a routine short-term toxicity test with an acceptable reproducibility. Physicochemical, biological, technological, as well as financial factors were considered. Details of this study which has led to the proposal outlined below are given in Vanhaecke *et al.* (1980). The technical description of the test follows the procedure outlined by the International Standardization Organization (ISO) for the acute toxicity tests on *Daphnia* and *Brachydanio*. It should be mentioned here that this proposal was

discussed in detail during a special workshop on toxicity tests with brine shrimp at the occasion of the "International Symposium on the Brine Shrimp *Artemia salina*" (Corpus Christi, Tex., August 1979).

According to the specialists present, a standardization of a simple routine test with *Artemia* nauplii, such as the one worked out by the scientists of the *Artemia* Reference Center, was highly desirable. At the end of the *Artemia* Symposium a recommendation was formulated that an intercalibration exercise based on this particular proposal should be organized at the international level. This exercise is in progress (1980-1981) in North America, under the joint supervision of the *Artemia* Reference Center at the State University of Ghent in Belgium and the Toxicology Section of the Freshwater Institute in Winnipeg, Canada.

An analogous exercise, sponsored by the Commission of the European Economic Communities, has also been started in Europe (in 1981) under the coordination of the *Artemia* Reference Center in Ghent, Belgium.

1. SCOPE AND FIELD OF APPLICATION

This standard method aims at determining the acute toxicity to nauplii of the brine shrimp *Artemia* of:

- a. chemical substances
- b. industrial and domestic effluents considered for dumping, or dumped into the marine environment.

2. PRINCIPLE

Determination of the concentration which kills 50% of the *Artemia* nauplii within 24 hr under the conditions described in the present standard. This concentration is known as the LC_{50} -24 hr.

3. LABORATORY

The preparation of the test, the storage of the dilution water, and all stages of the test procedure described below must take place in an atmosphere free from dust and toxic vapors.

4. MATERIALS

4.1. The Test Organism

A homogenous population of instar II-III nauplii hatched out from cysts of a well-defined *Artemia* strain must be used to carry out the test.² If other cysts are

² During the extensive study leading to this proposal (Vanhaecke *et al.*, 1980) it appeared that *Artemia* strains from different geographical origin often have different sensitivities. This finding is in agreement with the results of the "International Study on *Artemia*" which is devoted to the characterization of *Artemia* strains. From this study it clearly appears that geographic races of brine shrimp can differ in many features, e.g., biometrical, genetical, biochemical, nutritional, etc. (Sorgeloos *et al.*, 1979). Brine shrimp are used more and more for fundamental research, very often with cysts of unknown origin. A plea was made during the "International Symposium on the Brine Shrimp *Artemia salina*" (Corpus Christi, Tex., August 1979) that scientists should at least use the same reference cysts to calibrate their results. The *Artemia* Reference Center at the State University of Ghent has now agreed to act as the central distribution center for such reference *Artemia* cysts. The *Artemia* Reference Center is willing to extend this service for toxicity purposes for the benefit of increased standardization of this particular bioassay.

used, especially those of unknown origin, the specific procedure outlined hereunder might not be applicable.

4.2. Dilution Water

A standard artificial seawater of $35 \pm 1\text{‰}$ is used for the hatching as well as for the test. Whenever possible, the artificial salt mixture of Instant Ocean dissolved in distilled water shall be utilized. After aeration and stabilization for 24 hr the dilution water should have a pH of 8.0 ± 0.5 and the oxygen content should be at least 90% saturation. If necessary the pH should be adjusted with concentrated hydrochloric acid or sodium hydroxide. Before the water is used it should preferably be filtered through a $1\text{-}\mu\text{m}$ filter under vacuum.

4.3. Laboratory Equipment

The laboratory equipment needed consists of:

- constant temperature cabinet $25 \pm 1^\circ\text{C}$;
- glass petri dishes ($60 \times 12\text{ mm}$) with appropriate covers;
- Pasteur pipettes with smoothed openings;
- cylindrical (graduated cylinders) or preferably cylindroconical hatching tubes (diameter $\pm 35\text{ mm}$) with a content of at least 100 ml;
- dissolved oxygen meter;
- binocular dissection microscope;
- small airpump (aquarium pump);
- bulb or light tube;
- usual laboratory materials.

4.4. Reference Toxicant

The selected reference chemical is sodium lauryl sulfate (grade 98–102%). This compound is commonly used in surface tensiometry research.

5. PROCEDURE

5.1. Hatching and Preparation of the Nauplii

For each test approximately 100 mg of cysts is incubated in 100 ml seawater (section 3.2) in a cylindroconical tube or graduated cylinder at a temperature of $25 \pm 1^\circ\text{C}$ and with lateral illumination by a bulb or light tube (intensity of at least 500 lux). All the cysts and the hatching nauplii should be kept in continuous suspension by gentle aeration provided by a small air tube extending to the bottom of the hatching device. After 18 up to 24 hr the aeration is stopped and the nauplii, which concentrate at the bottom of the tube, are sucked out by pipetting and transferred into an Erlenmeyer flask containing 200 ml of seawater. The suspension should be gently aerated. The nauplii should be kept for exactly 24 hr at a temperature of $25 \pm 1^\circ\text{C}$. During that time they will all molt to the instar II and some of them to the instar III stage. At the end of this period an aliquot of the nauplii is poured into a petri dish for subsequent manual distribution to the test petri dishes.

5.2. *The Toxicity Test: General*

The test is carried out in small petri dishes. Ten nauplii are transferred with a Pasteur pipet into each dish. The volume of seawater carried over with the nauplii should be minimal. The dishes are filled with 10 ml of the respective concentrations of the toxicant, closed, and incubated in darkness at a temperature of $25 \pm 1^\circ\text{C}$. After 24 hr the number of dead larvae is counted in each petri dish. The nauplii are considered dead if no movement of the appendages is observed within 10 sec. Immediately after counting, the oxygen concentration is measured in the petri dish with the lowest concentration of toxicant that induced a 100% mortality.

5.3. *Preliminary Test*

This test is performed to determine the "critical range." A series of geometrically spaced concentrations or dilutions of the toxicant are prepared with artificial seawater. Example for chemical substances: 10,000, 1000, 100, 10, 0.1, 0.01 mg/liter; example for effluents: 100, 10, 1, 0.1, 0.01%. The preliminary test is carried out with only one petri dish per concentration. An additional dish with 10 nauplii in 10 ml artificial seawater is included as control.

5.4. *Definitive Test*

This test aims at the determination of the LC_{50} -24 hr on the basis of the critical range concentrations obtained in the preliminary test. Concentrations and dilutions are chosen from a logarithmic scale (Doudoroff *et al.*, 1951). In principle five concentrations should be sufficient. For a satisfactory LC_{50} , however, at least three data must be situated in the mortality range 5-95%. If this is not the case, the test should be repeated with additional concentrations from the dilution scale. For each concentration, including the control, three replicates should be used.

5.5. *Checking of the Sensitivity of the Artemia Nauplii and of the Conformity of the Experimental Procedure*

The LC_{50} -24 hr of the reference chemical sodium lauryl sulfate must be determined each time in parallel with the definitive test in order to verify the stability of the sensitivity of the experimental procedure. The following concentrations of sodium lauryl sulfate should be tested in three replicates: 10, 13.5, 18, 24, and 32 mg/liter. To prepare the sodium lauryl sulfate solution it is recommended to use a magnetic stirrer, since this product does not dissolve quickly.

6. CALCULATION AND VALIDITY OF THE RESULTS

The LC_{50} -24 hr can be calculated by graphical interpolation. The percentages of mortality between 5 and 95% are calculated from the average number of dead nauplii per concentration, and plotted on log-probit paper. A straight line is drawn at sight through the points. The intersection of this line with the 50% mortality horizontal line determines the LC_{50} -24 hr. An alternative more precise procedure is to use the method of Litchfield and Wilcoxon (1949) with which the 95% confidence limits can be calculated. The test can be considered valid if the following

conditions are fulfilled:

- the percentage mortality in the control does not exceed 10%;
- the LC_{50} -24 hr of sodium lauryl sulfate is situated between 13.3 and 19.9 mg/liter;
- the dissolved oxygen concentration at the end of the experiment is higher than 2 mg/liter in the lowest concentration with 100% mortality of the larvae.

7. REPORTING OF THE RESULTS

The following facts shall always be reported:

- the origin of the *Artemia* strain and, if possible, the batch number of the commercial brand used;
- the calculated LC_{50} -24 hr if possible with the 95% confidence limits;
- the critical (0-100% mortality) range;
- the data confirming the validity of the results:
 - (a) LC_{50} -24 hr of sodium lauryl sulfate,
 - (b) percentage mortality of the controls,
 - (c) O_2 concentration at the end of the test;
- the method of calculation used for the determination of the LC_{50} -24 hr;
- any deviation of the standard procedure and any problem encountered during the test.

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